Pergamon

PII: S0146-6380(97)00114-9

Org. Geochem. Vol. 28, No. 1/2, pp. 11–26, 1998

Printed in Great Britain
0146-6380/98 $19.00 + 0.00

Organic geochemical studies of soils from the Rothamsted Classical Experiments—II, Soils from the Hoosfield Spring Barley Experiment treated with different quantities of manure

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(Received 8 July 1997; returned to author for revision 29 July 1997; accepted 30 October 1997)

Abstract—Total lipid extracts (TLEs) were obtained from soil samples taken in the years 1882, 1913, 1946, 1965 and 1995 from three treatments of the Hoosfield Spring Barley Experiment at Rothamsted Experimental Station, Harpenden, Hertfordshire, U.K. The extracts were fractionated and molecular analyses performed using gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). In addition to the soil samples (contemporary and archived), the two primary organic inputs, barley and farmyard manure (FYM), were studied so that the composition and diagenetic behaviour of extractable lipids from the two inputs could be assessed. The major aliphatic soil lipids exhibited variable dominance with respect to the expression of barley and FYM derived lipids. Wax esters were of low abundance and too strongly affected by degradation and transesterification processes to identify a dominant input whilst the composition of soil *n*-alkanols was largely determined by FYM with a minor pedogenic input. *n*-Alkanoic acids increased in overall abundance in soils with a continual FYM input and showed appreciable degradation in soils receiving no manure. C_{32} $\beta\beta$ hopanoic acid was detected in two plots and appeared to degrade at a rate similar to 5β-stanols with the most likely source of this compound being the FYM. Measurements of absolute concentrations of 5β-stanols, biomarkers characteristic of manuring, revealed that a manuring signal persisted for >120 years within the soil which had been intensively cultivated annually and had received no manure since 1871. The persistence of a manure signal in soils has important implications for archaeological studies of agricultural practices based on 5β-stanols. © 1998 Elsevier Science Ltd. All rights reserved

Key words—lipids in soil, stanols, manure, soil, gas chromatography, mass spectrometry

INTRODUCTION

Molecular studies of soil organic matter are relatively scarce with the majority of investigations focusing on bulk properties. Of the studies that have been undertaken a large number deal solely with high molecular weight components, such as humic acids (Martin *et al.*, 1979; Martin and Gonzalez-Vila, 1983; Kögel-Knabner *et al.*, 1988; Schulten and Hempfling, 1992; Hatcher and Clifford, 1994; Richnow *et al.*, 1994; del Rio *et al.*, 1994) whilst rather less attention has focused on low molecular weight components such as lipids. Since a number of reviews are available covering earlier studies of soil lipids (e.g. Morrison, 1969; Braids and Miller, 1975; Dinel *et al.*, 1990) the short overview given below will focus mainly on more recent reports.

Soil lipids generally constitute 1–5% of the total soil organic matter, consisting predominantly of *n*-alkanes, *n*-alkanols, wax esters, fatty acids, steroids, triterpenoids and acyl glycerols. They originate from both plants and animals as products of deposition, decomposition and exudation, as well as from various pedogenic sources, including fungi, bacteria and mesofauna. The chemical components of any soil are strongly affected by environmental conditions which are determined primarily by local climate, geology and vegetation cover. The composition, concentration and diagenetic fate of soil lipids will vary between different environments due to differences in the source of the organic compounds and the change in inorganic mineral components (Miller and Donahue, 1995). It has been shown that a relatively greater lipid content is found in soils possessing low microbiological activity, whilst soil pH also affects lipids, with strongly acidic environments exhibiting higher lipid contents (Fridland, 1976). Lipids can exist in a number of microenvironments within the soil being either free entities in the soil matrix, chemically bound components of humic material or physically adsorbed to clay particles (Jambu *et al.*, 1978). In the latter case, as the proportion of clay particles

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decreases so the amount of lipid, relative to humic material, has been found to increase; the concentration and microenvironment of aliphatic soil lipids can also affect the stability of soil aggregates (Dinel *et al.*, 1992).

It has been observed that decay resistant compounds associated with surface vegetation have the greatest significance within the profile of non-anthropogenic soils; the proportion derived from the *in situ* activity of micro-organisms being comparatively smaller (Fridland, 1982).

Even so, the presence of certain micro-organisms can be detected through the use of specific biomarkers. Bacteriohopanetetrol occurs as a membrane constituent in prokaryotes (Rohmer *et al.*, 1984) and has been shown to occur in some soils, as have related diagenetic degradation products (Ries-Kautt and Albrecht, 1989). The presence of these compounds has been ascribed to bacterial activity which is directly affected by pH, moisture and plant type. Minor bacterial population increases have also been reported as the result of manure application (Eiland, 1980).

The majority of studies concerning soil lipids, including some of the above, have focused on the input of vegetation derived lipids to the soil and/or the subsequent products of diagenesis. For example, some research has centred on investigations of the lipids associated with peat. The relatively slow rate of degradation in peat, due to acidity and anaerobicity in lower waterlogged layers, provides an ideal environment for the preservation of vegetation derived lipids (Amblès *et al.*, 1989). Farrimond and Flanagan (1996) were able to differentiate between various plant inputs (trees *vs* shrubs and moss) to a mid-Holocene peat by analysing the distributions of *n*-alkane and *n*-alkanols and comparing the results with those obtained from pollen analysis.

Another area of interest is agricultural soil although, as stated earlier, investigations on a molecular level are relatively scarce. Wang *et al.* (1971) observed characteristic chromatographic peaks corresponding to unknown compounds present in a soil cultivated with sugar cane (*Saccharum officinarum*); this enabled it to be differentiated from soils cultivated with other crops. In other studies, the expression of manure within the lipid profile of an agricultural soil, relative to samples of unmanured soil, has been demonstrated through the detection of characteristic 5β-stanols amongst the free soil lipids (Evershed and Bethell, 1996; Evershed *et al.*, 1997; Simpson *et al.*, in press). Whilst potentially prone to greater degradation effects (cf. peat), it is not unreasonable to expect a chemical signature indicative of former land use to persist within the pedological record.

Obtaining insights into changes in vegetation or land use is especially significant within an archaeological context. Knowledge of processes once affecting the soil at an archaeological site may provide valuable information concerning the agricultural activities practiced by ancient man. Changes in crop, or natural vegetation, are also an important aspect. Indeed it is not unreasonable to expect the environment surrounding an archaeological site to have altered during the period of occupation with changes in soil organic matter composition providing potential opportunities to utilise organic geochemical techniques to monitor the impact of changes in vegetation and/or land-use. One important aspect is the detection of waste disposal and manuring which provides insights into agricultural practices and site limits (Bethell *et al.*, 1994; Evershed and Bethell, 1996; Evershed *et al.*, 1997). A number of methods have already been utilised to detect ancient manuring practices. These include the use of elemental data (Provan, 1973), phosphorous concentration (Eidt, 1984; Prøsch-Danielsen and Simonsen, 1988), micromorphology (Limbrey, 1975), potsherd scatter (Courty *et al.*, 1989; Bintliff and Snodgrass 1988) and magnetic susceptibility (Mullins, 1977). The use of highly diagnostic decay resistant biomarkers offers a potentially robust and direct means of detecting manure inputs into archaeological soils.

Soils obtained from the Rothamsted Experimental Station, provide a unique source of material for the study of soil organic geochemical processes related to changing land-use. The various agricultural plots and other areas have associated records detailing agricultural practices and soil treatments. Additionally, archived samples from a series of long term experiments, dating over 150 years, are available which provide opportunities for various time-course analyses. We have already undertaken one investigation of soils and vegetation from the Broadbalk Wilderness (van Bergen *et al.*, 1997) in order to study the effects of reversion from cultivation of cereals to woodland and grassland. The investigation showed that while low molecular weight soil lipids were dominated by compounds derived from the overlying vegetation, pyrolysis data from the corresponding high molecular weight fractions did not reflect the vegetation composition. The lack of a high molecular weight signature was ascribed to rapid diagenesis influenced by the slightly alkaline pH of the soil thereby emphasising the significance of soil lipid analysis.

The aim of this research was to investigate the effect that continuous cultivation of barley and the variable application of manure have had on the free lipid content of soil. The study is particularly concerned with the long-term behaviour of specific biomarkers related to micro-organisms and the aforementioned inputs, over a long period of time. The results can then be used to provide information on the pedological survival of these molecules

which can be utilised in archaeological and environmental studies.

SAMPLE DESCRIPTION

The samples studied were from the Hoosfield Spring Barley experiment at Rothamsted Experimental Station in southeast England. The soil is a Stagnogleyic paleo-argillic brown earth, with a loamy surface layer overlying Clay-with-flint; Batcombe Series (Jenkinson and Johnston, 1977). It can be further classified as a Chromic Luvisol (F.A.O., 1990) or as an Aquic Paleudalf (U.S.D.A., 1992).

The Hoosfield Spring Barley experiment was started in 1852 thereby making it the longest running cereal experiment in the world with the exception of the adjacent Broadbalk Wheat experiment. It consists of a number of large, unreplicated strips receiving different combinations of phosphorous (P), potassium (K) and magnesium (Mg). Each strip was originally divided at right angles to test various forms of nitrogen (N), this test was discounted in 1966. Additional plots included a farmyard manure (FYM) treatment which was divided after 20 years to test the effect of FYM residues. Since 1968 each main plot has been divided to test four rates of inorganic N. Fuller details are given by Warren and Johnston (1967), Jenkinson and Johnston (1977) and the Guide to the Classical Field Experiments (Anon, 1991).

Soils from three different treatments were used in this study: one has received no manure since 1852 (unmanured); one received FYM for 20 years from 1852 to 1871 but none since (FYM-residues); the third has received FYM each year since 1852 (continuous FYM). Treatment details are given in Table 1. Fresh soils were sampled in May 1995 to a depth of 23 cm using a 2-cm diameter auger. Air-dried, archived soils from 1882, 1913, 1946 and 1965 were also sampled. These were stored in large, corked bottles (~2.5 l) except those from 1965 which were stored in paper bags in cardboard boxes. The archived soil was also taken to a depth of 23 cm. A dried, finely ground sample of the FYM that had been applied in autumn 1994 was obtained together with a fresh sample of the standing barley crop.

EXPERIMENTAL

Sample preparation and solvent extraction

Fresh soil and vegetation samples were all oven dried at 60°C. Soil samples were then partially crushed with a pestle and mortar and, subsequently, sieved using 2 mm and 75 μm sieves. Dried vegetation samples were crushed using the same method but liquid nitrogen was added to facilitate the process. All soil samples were Soxhlet extracted for 24 hr using dichloromethane/acetone (9:1 v/v) to obtain a total lipid extract (TLE). Vegetation samples were ultrasonically extracted (4×) with dichloromethane/acetone (9:1 v/v).

The TLEs obtained from the Hoosfield samples were further fractionated initially into two fractions using an aminopropyl bond-elute cartridge. The first fraction comprised neutral lipids whereas the second contained predominantly fatty acids. The former fraction was separated into a further five fractions *via* gradient elution on a silica flash column. Urea adduction of the "alcohol" fraction yielded two fractions, one predominantly *n*-alkanols and the other sterols. Finally, 5β-stanols were isolated from the total sterol fraction by thin layer chromatography (TLC); isolating a band determined by the R_f value corresponding 5β-cholestan-3β-ol (**1a**; 0.48). TLC plates were developed using ethyl acetate in hexane (20% v/v) as eluent.

Derivatization

All fractions except the hydrocarbons were derivitized by heating sample aliquots with 30 μl of *N,O*-bis(trimethylsilyl)trifluroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS), at 70°C for 1 hr. 5β-stanol fractions were heated for 12 hr to ensure complete formation of the corresponding trimethylsilyl ethers.

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS)

GC analyses were performed using a Hewlett-Packard 5890 series II gas chromatograph fitted

Table 1. Details regarding sample codes and various soil treatments

Original Plot No.[a]	Current Plot No.[b]	Treatment Code[c]	Description[d]
1.0	111	Unmanured	No manure or PK fertilizer since 1852
7.1	711	FYM-residues	35 t ha^{-1} yr^{-1} FYM 1852-1871, none since
7.2	721	Continuous FYM	35 t ha^{-1} yr^{-1} FYM since 1852

[a]Main plots were originally 42 × 17.5 m (1.0) or 42 × 11.4 m (7.1, 7.2)
[b]Following the introduction of wider discards and divisions to test four rates of N, sub-plots are now 10.5 × 12.2 m (111) and 10.5 × 9.1 m (711, 721)
[c]All treatments have received inorganic N since 1968 (calcium ammonium nitrate until 1985, ammonium nitrate since) supplying an average 72 kg N ha^{-1} yr^{-1}
[d]35 t_{FYM} ha^{-1} contains ca. 20% dry matter and ca. 235 kg N ha^{-1}.

with a fused silica capillary column (50 m × 0.32 mm) coated with a 100% dimethyl polysiloxane stationary phase (CPSil-5 CB, film thickness 0.12 μm). Derivatized samples were injected (1.0 μl) *via* an on-column injector as solutions in hexane. The temperature was programmed from 40°C (1 min) to 200°C at a rate of 10°C min^{-1} then to 300°C at a rate of 3°C min^{-1} with a final time of 20 min. Hydrogen was used as carrier gas for all samples except the hydrocarbon fraction when helium was used as carrier gas in order to facilitate resolution of the internal standard. GC analyses of wax ester fractions were made using a column capable of performing at elevated temperature (DB1, 15 m × 0.32 mm; 0.1 μm film thickness; He carrier gas). The temperature was programmed from 50°C (2 min) to 350°C at a rate of 10°C min^{-1} with a final time of 10 min. The majority of GC/MS analyses were performed using a Varian 3400 gas chromatograph fitted with a 50 m fused silica capillary column (as above) and a temperature programmable injector (SPI) coupled, *via* a heated transfer line, to a Finnigan MAT TSQ700 triple quadrupole mass spectrometer. The mass spectrometer was operated in single quadrupole mode, scanning the third quadrupole in the range of *m/z* 50 to 650 with a cycle time of 1 s. Electron ionization was performed with an electron energy of 400 μA; the ion source was maintained at a temperature of 170°C. GC/MS analyses of wax ester fractions were made using a Carlo Erba 5160 GC equipped with a DBI high temperature column (above) and on-column injector coupled, *via* a heated transfer line, to a Finnigan MAT 4500 quadrupole mass spectrometer scanning the range *m/z* 50 to 850 with a cycle time of 1.5 s. The GC oven temperature was programmed from 50°C (2 min) to 350°C at a rate of 10°C min^{-1} with a final time of 10 min. Electron energy was maintained at 300 μA with an ion source temperature of 190°C. Both mass spectrometers were operated with an electron voltage of 70 eV.

RESULTS

The Hoosfield Spring Barley experiment has a well-documented history spanning 144 years. Since the experiment started, there have been only two primary extra-pedological sources of lipid to the soils studied, namely the stubble and roots of the barley crop and FYM. FYM may have been applied to the site prior to the experiment starting but amounts are likely to have been small. Initial analyses were conducted on these two inputs to identify characteristic lipid components and provide the starting point for monitoring changes in the soil lipid composition during the course of the experiment. In the text numbers in bold refer to Fig. 4 and Appendix A.

Analysis of soil inputs—barley

The non-polar hexane fraction, obtained from the extract of the modern barley crop is dominated by *n*-alkanes ranging in carbon number from C_{19} to C_{35}. The distribution maximises at C_{33}, however, overall describes a skewed bimodal pattern due to a slightly elevated abundance of the C_{25} component. Wax ester constituents of the TLE elute in the dichloromethane fraction and are shown in Fig. 1a. The observed distribution ranges from C_{36} to C_{60} with the C_{42} to C_{48} homologues predominating. There is no single maxima because the C_{42} to C_{48} components are of almost equal abundance and are almost exclusively based on a C_{26} *n*-alkanol moiety. The isolated free *n*-alkanols exhibit a much narrower distribution ranging from C_{22} to C_{28} (Fig. 2a). A peak corresponding to *n*-hexacosanol (C_{26}) dominates (13620 μg g^{-1} dwt) and more minor peripheral *n*-alkanol components yield a monomodal distribution. Even carbon number *n*-alkanoic acids are observed ranging from C_{12} to C_{34} (Fig. 3a) with a maximum at C_{16}. However, the latter part of the homologous series is clearly dominated by *n*-hexacosanoic acid (C_{26}) thereby producing a bimodal distribution. $C_{18:2}$ diunsaturated and $C_{18:3}$ triunsaturated acids are also detected in relatively high abundance. Analysis of the isolated sterol fraction (Fig. 4a) reveals a chromatogram dominated by 24-ethylcholest-5-en-3β-ol (**4c**); cholest-5-en-3β-ol (**4a**), 24-methylcholest-5-en-3β-ol (**4b**), 24-ethylcholest-5,22-dien-3β-ol (**4e**), 24-methyl-5α-cholestan-3β-ol (**3b**) and 24-ethyl-5α-cholestan-3β-ol (**3c**) are also present in lower concentrations. Only a proportion, perhaps 25% of those components measured in the whole crop, would have been incorporated into the soil as roots and stubble.

Analysis of soil inputs—FYM

The non-polar FYM hexane fraction is also dominated by a series of *n*-alkanes which range from C_{21} to C_{35}. The peaks corresponding to odd numbered *n*-alkanes describe a monomodal distribution maximising at C_{31}. The results obtained from the dichloromethane fraction reveal wax esters ranging from C_{36} to C_{60} with two maxima at C_{48} and C_{52} (Fig. 1b); as with barley each wax ester component is almost solely based on the C_{26} *n*-alkanol. No homologue is significantly more abundant than any other. The *n*-alkanol (Fig. 2b) fraction exhibits a narrow monomodal distribution ranging from C_{22} to C_{30} and maximising at *n*-hexacosanol (C_{26}); this component is remarkably more abundant than the other *n*-alkanol homologues although not to the same extent as in the barley extract. The distribution of *n*-alkanoic acids is wide, beginning with dodecanoic acid (C_{12}) and increasing to hexatriacontanoic acid (C_{36}; Fig. 3b). The series is dominated by hexacosanoic acid (C_{26}) although hexadecanoic acid (C_{16}) is an important component

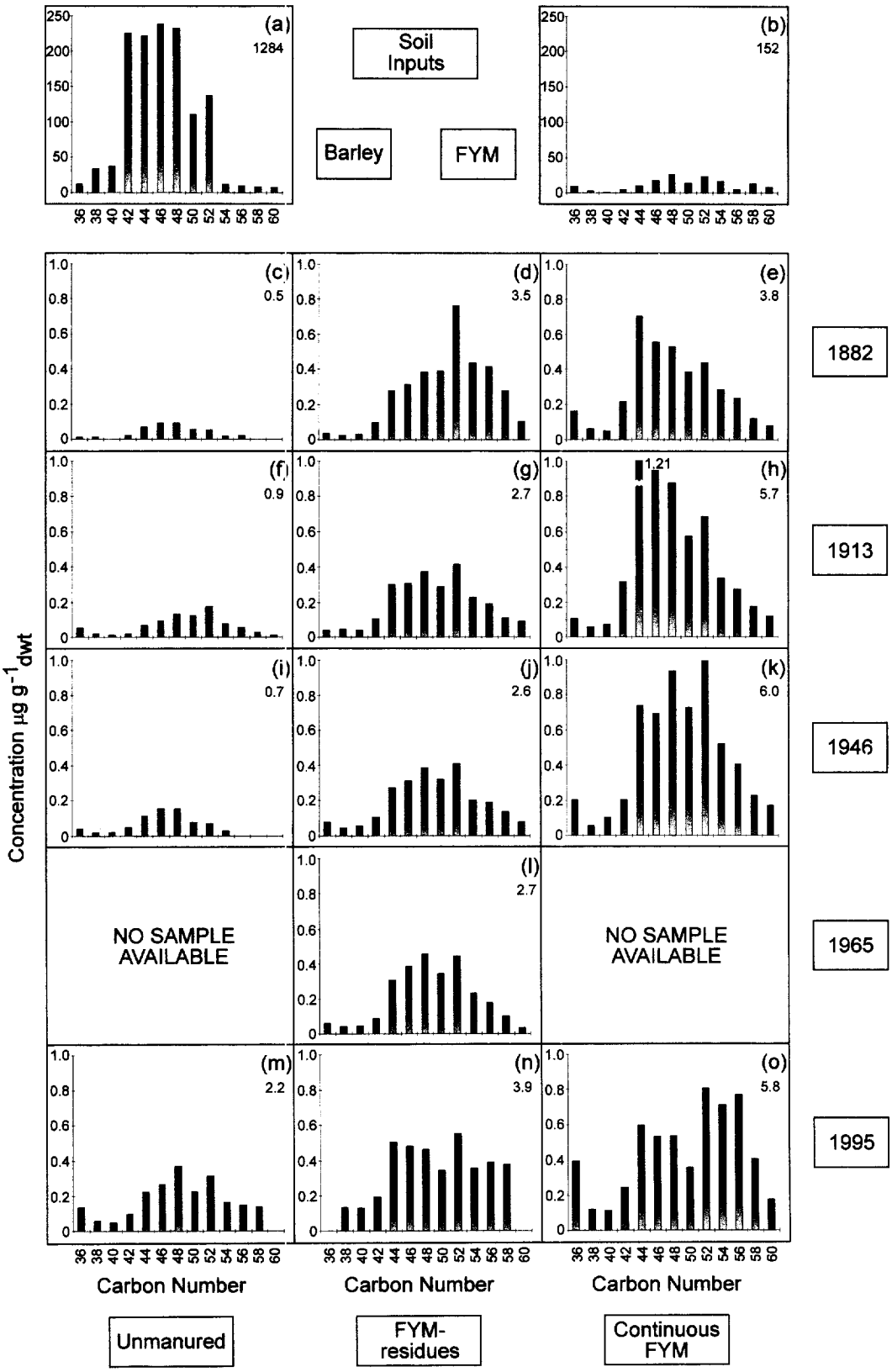

Fig. 1. Distributions for the major wax ester components detected in the inputs and soil samples. Each column relates to a different treatments whilst each row corresponds to a specific sampling year. The value below each figure letter, (a) to (o), denotes total abundance ($\mu g\ g^{-1}_{soil\ dwt}$).

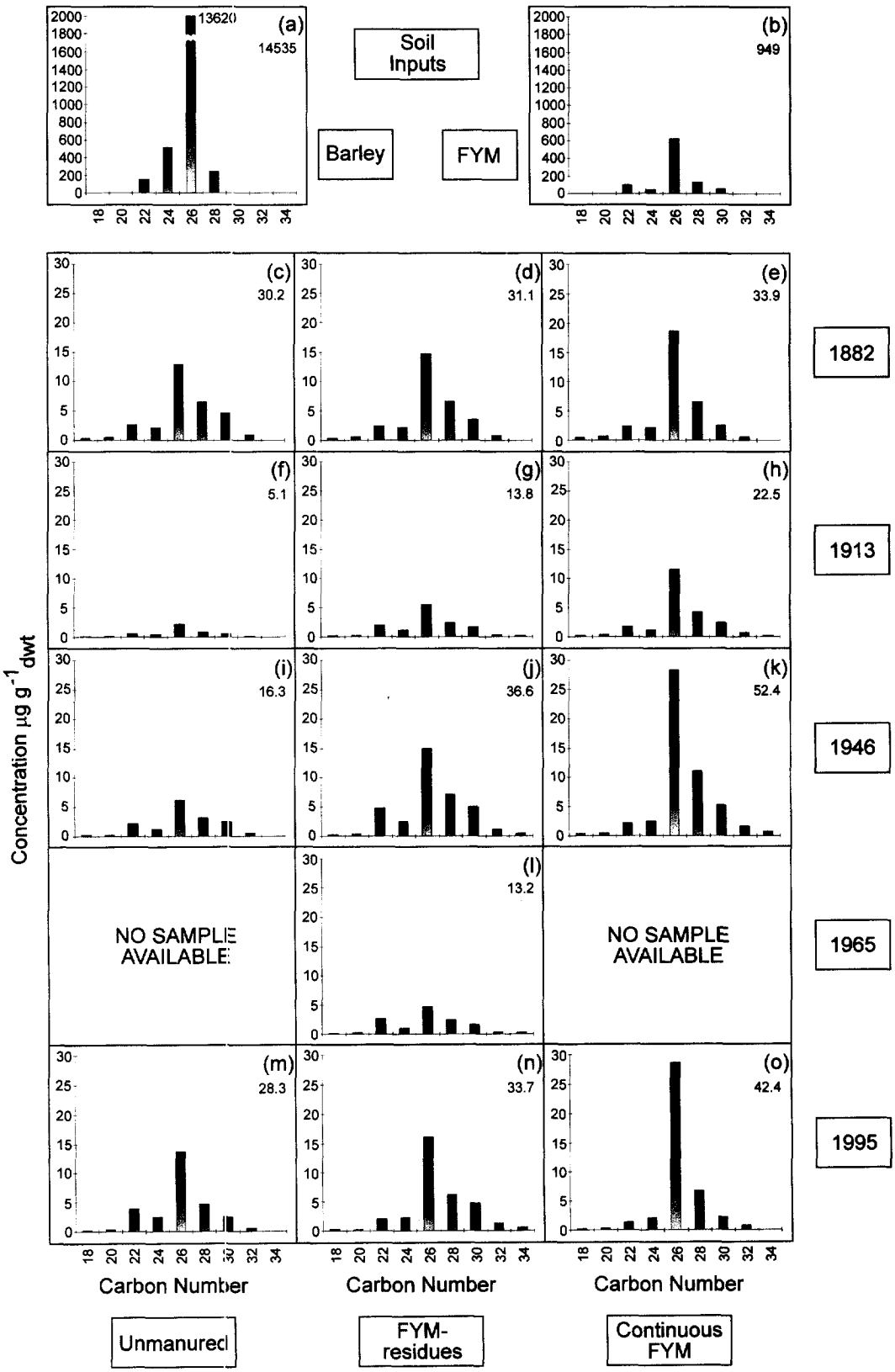

Fig. 2. Distributions for the major *n*-alkanol components detected in the inputs and soil samples. Each column relates to a different series whilst each row corresponds to a specific sampling year. The value below each figure letter, (a) to (o), denotes total abundance ($\mu g\ g^{-1}_{soil\ dwt}$).

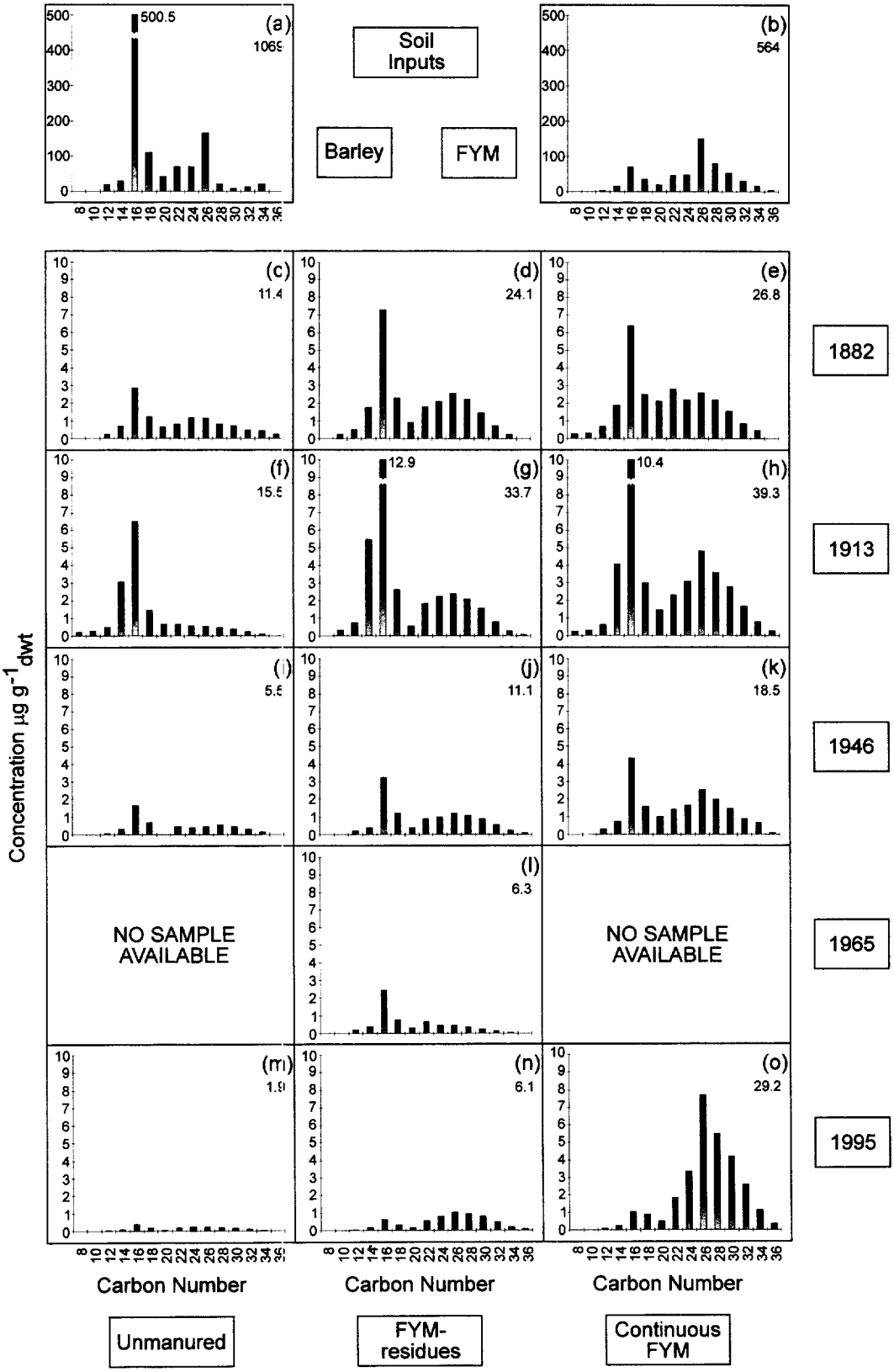

Fig. 3. Distributions for the major even-fatty acid components detected in the inputs and soil samples. Each column relates to a different treatment whilst each row corresponds to a specific sampling year. The value below each figure letter, (a) to (o), denotes total abundance ($\mu g\ g^{-1}_{soil\ dwt}$).

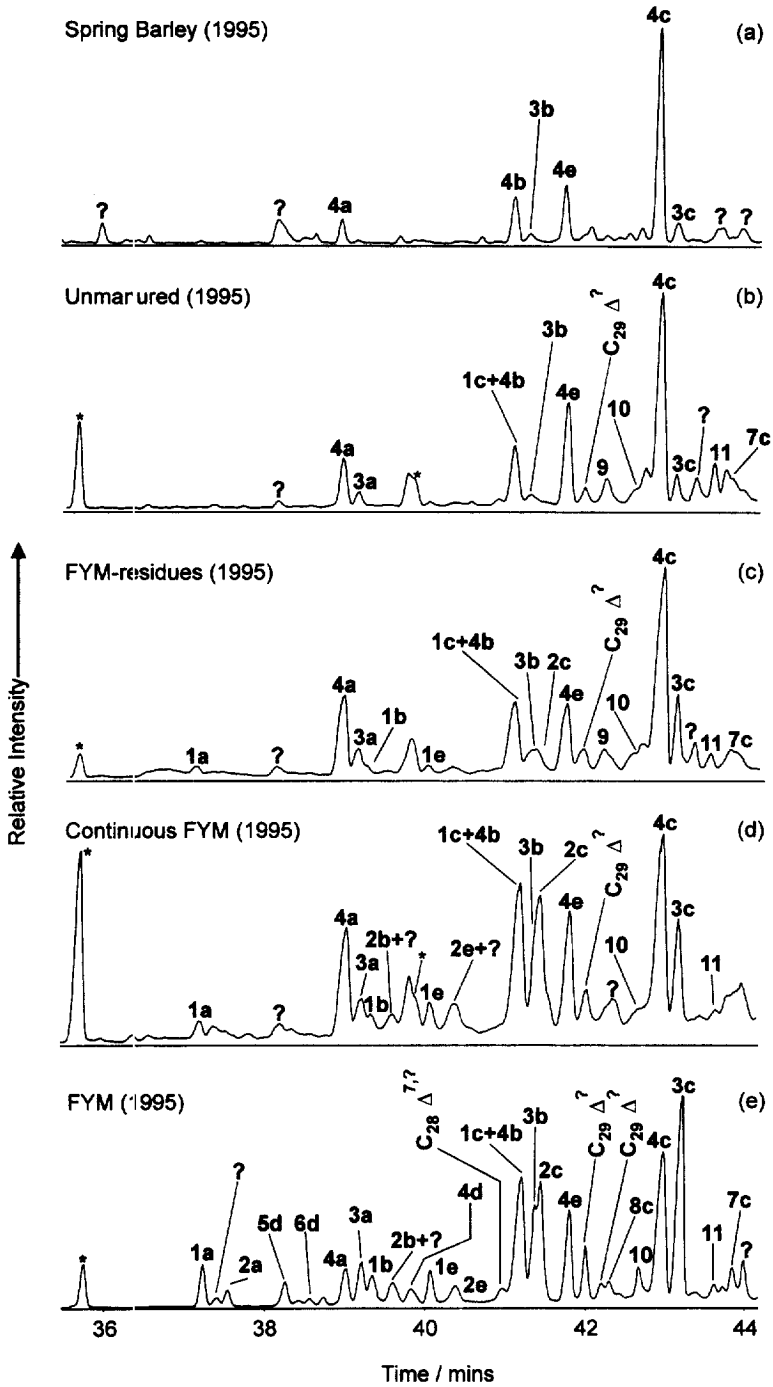

Fig. 4. Partial GC-FID traces of the sterol fractions (urea "non-adducts") from the 1995 samples. Peak assigments are: 5β-cholestan-3β-ol (**1a**), 24-methyl-5β-cholestan-3β-ol (**1b**), 24-ethyl-5β-cholestan-3β-ol (**1c**), 24-ethyl-5β-cholest-22-en-3β-ol (**1e**), 5β-cholestan-3α-ol (**2a**), 24-methyl-5β-cholestan-3α-ol (**2b**), 24-ethyl-5β-cholestan-3α-ol (**2c**), 24-ethyl-5β-cholest-22-en-3β-ol (**2e**), 5α-cholestan-3β-ol (**3a**), 24-methyl-5α-cholestan-3β-ol (**3b**), 24-ethyl-5α-cholestan-3β-ol (**3c**), cholest-5-en-3β-ol (**4a**), 24-methyl-cholest-5-en-3β-ol (**4b**), 24-ethylcholest-5-en-3β-ol (**4c**), 24-ethylcholest-5,22-dien-3β-ol (**4e**), 24-methyl-5β-cholest-7,22-dien-3β-ol (**5d**), 24-methyl-5β-cholest-7,22-dien-3α-ol (**6d**), 24-ethylcholest-4-en-3-one (**7c**), 24-ethyl-5β-cholestan-3-one (**8c**), taraxerol (**9**), β-amyrin (**10**), lupeol (**11**). A proportion of unlabelled peaks (*) represent *n*-alkanols "carried-over" in the urea adduction procedure. For structures see Appendix A.

amongst the early eluting homologues. The slightly higher abundance of the C_{22} component causes the overall shape to deviate from a perfect monomodal distribution of latter components ($>C_{18}$). Homologues ranging from C_{13} to C_{33} are observed in the normal odd-chain fatty acid distribution and are dominated by long-chain homologues centred about the C_{25} component. C_{32} $\beta\beta$-hopanoic acid (**12**) is also present at low concentration (2239 ng g^{-1} dwt). The sterol fraction is complex and contains a large number of compounds (Fig. 4e). Cholest-5-en-3β-ol (**4a**), 24-methyl-cholest-5-en-3β-ol (**4b**), 24-ethyl-cholest-5,22-dien-3β-ol (**4e**) and 24-ethyl-cholest-5-en-3β-ol (**4c**) are all present at relatively high concentration with the latter component exhibiting the highest abundance of the four. Also present are the associated stanols: 5β-cholestan-3β-ol (**1a**), 5β-cholestan-3α-ol (**2a**), 5α-cholestan-3β-ol (**3a**) and the corresponding C_{28} and C_{29} carbon number homologues of these (**1b, 2b, 3b, 1c, 2c, 3c**); 24-ethyl-5α-cholestan-3β-ol (**3c**) is the dominant peak in the chromatogram. Minor quantities of 24-methyl-5β-cholest-7,22-dien-3β-ol (**5d**), 24-methyl-5β-cholest-7,22-dien-3α-ol (**6d**), 24-ethyl-5β-cholest-22-en-3β-ol (**1e**) and 24-ethyl-5β-cholest-22-en-3α-ol (**2e**) are also present, as is the reduction intermediate 24-ethyl-5β-cholestan-3-one (**8c**; Ren *et al.*, 1996). The triterpenol components taraxerol (**9**), β-amyrin (**10**) and lupeol (**11**) are also observed to occur in low abundance.

Analysis of the soil extracts

Hydrocarbon fractions obtained from the archived samples yield results indicative of petroleum contamination, most likely from paraffin wax used to seal the corks in the jars used for storage. The hydrocarbon distributions of the modern day (1995) soil samples from the different plots are very similar. For each, a range of *n*-alkanes from C_{19} to C_{35} are identified with the most abundant components being the C_{27}, C_{29}, C_{31} and C_{33} homologues which exhibit a C_{31} maxima.

Wax esters in the soil samples (Fig. 1c–o) range from C_{36} to C_{60} with a number of other homologues present at trace level; the C_{42} to C_{56} homologues generally dominate the distributions. For the FYM-residues and Continuous FYM soils the total abundance of wax esters varies little with time, however, that of the Unmanured soil increases. Within any one year total concentration is in the order Continuous FYM > FYM-residues > Unmanured. Each distribution is skewed towards the higher homologues with increasing time. It is interesting to note that the C_{50} components are slightly less abundant than adjacent homologues in all but the first three Unmanured and initial FYM-residues samples. Unlike the two inputs no *single* alkanol moiety dominates the wax-ester compositions; the alkanol moieties vary in the range *n*-C_{24} to *n*-C_{32}, although the *n*-C_{26} component is often pronounced.

Analysis of the *n*-alkanol fractions reveals a series of distributions which exhibit very similar relative abundances (Fig. 2c–o). The homologues show monomodal distributions in the C_{18} to C_{34} range always maximising about *n*-hexacosanol (C_{26}). The same trends are seen in the concentrations of *n*-alkanols, with varying manure treatments, as are observed for the wax esters.

The even *n*-alkanoic acid components of the soils range between C_8 and C_{36} (Fig. 3c–o). In this case a steady decrease in the abundance of the *n*-alkanoic acids in extracts from the Unmanured and FYM-residues treated soils is clearly evident. The overall abundance of *n*-alkanoic acids in the Continuous FYM soil does not decrease with time and there is an overall increase in the abundance of the higher carbon number homologues. Again, the trend in concentration (Unmanured < FYM-residues < Continuous FYM) for a single year is clearly discernible. The trends in the distributions of odd-chain *n*-alkanoic acids parallel those observed for the even-chain homologues, except for the Continuous FYM soil, where there is a slight decrease in overall abundance with time. In addition to the *n*-alkanoic acids each sample contains various branched-chain components; predominantly the *iso*- and *anteiso*-isomers. The C_{15} homologues of these two isomers are present in all samples except the Unmanured soils from 1913 and 1995 whilst other *iso*- and *anteiso*-homologues are more sporadic in occurrence. C_{32} $\beta\beta$ hopanoic acid (**12**) is detected in the samples from the FYM-residues and Continuous FYM soils (Fig. 5) with the concentration of this component fluctuating between 0.1 and 0.2 $\mu g\, g^{-1}_{dwt}$ in the Continuous FYM soils, whilst in the FYM-residues treatment it gradually drops in concentration from 0.19 to 0.05 $\mu g\, g^{-1}_{dwt}$. The Unmanured soils do not contain this compound at detectable levels.

The sterol constituents of all three soils are dominated by cholest-5-en-3β-ol (**4a**), 24-methylcholest-5-en-3β-ol (**4b**), 24-ethylcholest-5,22-dien-3β-ol (**4e**) and 24-ethylcholest-5-en-3β-ol (**4c**; Fig. 4b, c and d); the latter compound is the major component in all samples analysed. The most significant differences in the steroidal lipids concern the abundance of the saturated stanols 5β-cholestan-3β-ol (**1a**), 5β-cholestan-3α-ol (**2a**), 5α-cholestan-3β-ol (**3a**) and the corresponding C_{28} and C_{29} carbon number homologues of these (**1b**, **2b**, **3b**, **1c**, **2c**, **3c**). Soil treated with manure, either continuously or for the first 20 years of the experiment only, exhibits higher concentrations of the 5β-stanol components, especially 24-ethyl-5β-cholestan-3β-ol (**1c**) (Fig. 4c and d). In contrast, soil which has been unmanured since at least 1852 contains low concentrations of these compounds (Fig. 4b). The higher plant triterpenols

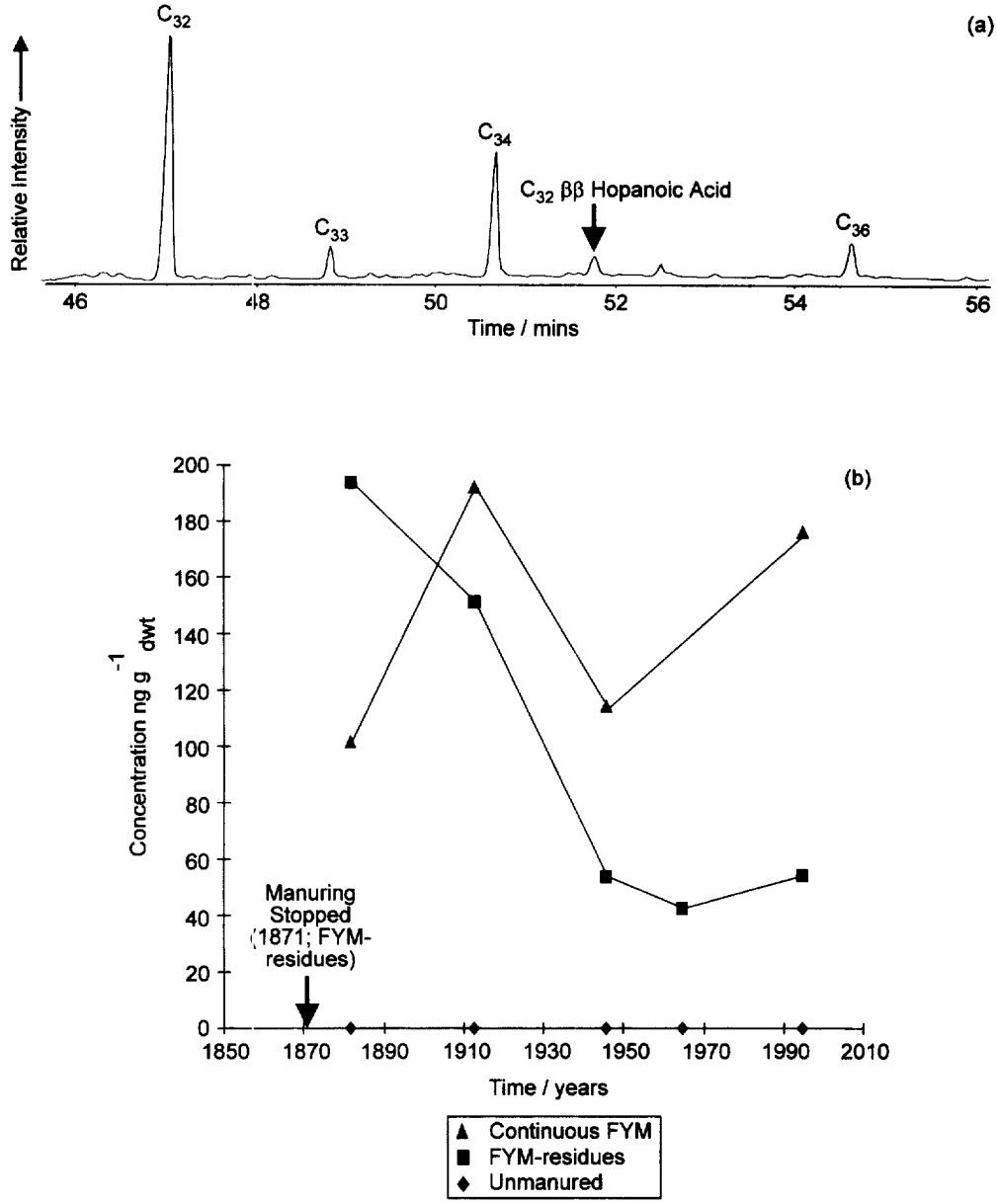

Fig. 5. Schematics depicting: (a) a partial gas chromatogram of the 1995 FYM-residues soil fatty acid fraction, and (b) the absolute concentration of C_{32} $\beta\beta$ hopanoic acid (**12**) in each of the soil samples analysed *vs* time.

taraxerol (**9**), β-amyrin (**10**) and lupeol (**11**) were detected in the majority of samples irrespective of treatment. The variation in concentration of the major 5β-stanol component (determined by GC following TLC separation), 24-ethyl-5β-cholestan-3β-ol (**1c**; Fig. 6a), with sampling year provides an important insight into the longevity of this compound in the Rothamsted soils. A general "background" of the compound, observed for the Unmanured soil, oscillates between 16 ng $g^{-1}_{soil\ dwt}$ and 37 ng $g^{-1}_{soil\ dwt}$. The FYM-residues soil, however, exhibits concentrations of 24-ethyl-5β-cholestan-3β-ol (**1c**) above that of the Unmanured soil despite apparent degradation with time. Remarkably, the value for the 1995 sample, at 64 ng $g^{-1}_{soil\ dwt}$, still exceeds the corresponding Unmanured "background". From the Continuous FYM soil it can be seen that the annual application of manure has a pronounced effect on the abundance of this component; the concentration of 24-ethyl-5β-cholestan-3β-ol (**1c**) increasing greatly to 464 ng $g^{-1}_{soil\ dwt}$. An analogous study of the C_{27} and C_{29} 5α-stanol (**3a** and **3c**) components shows an over-

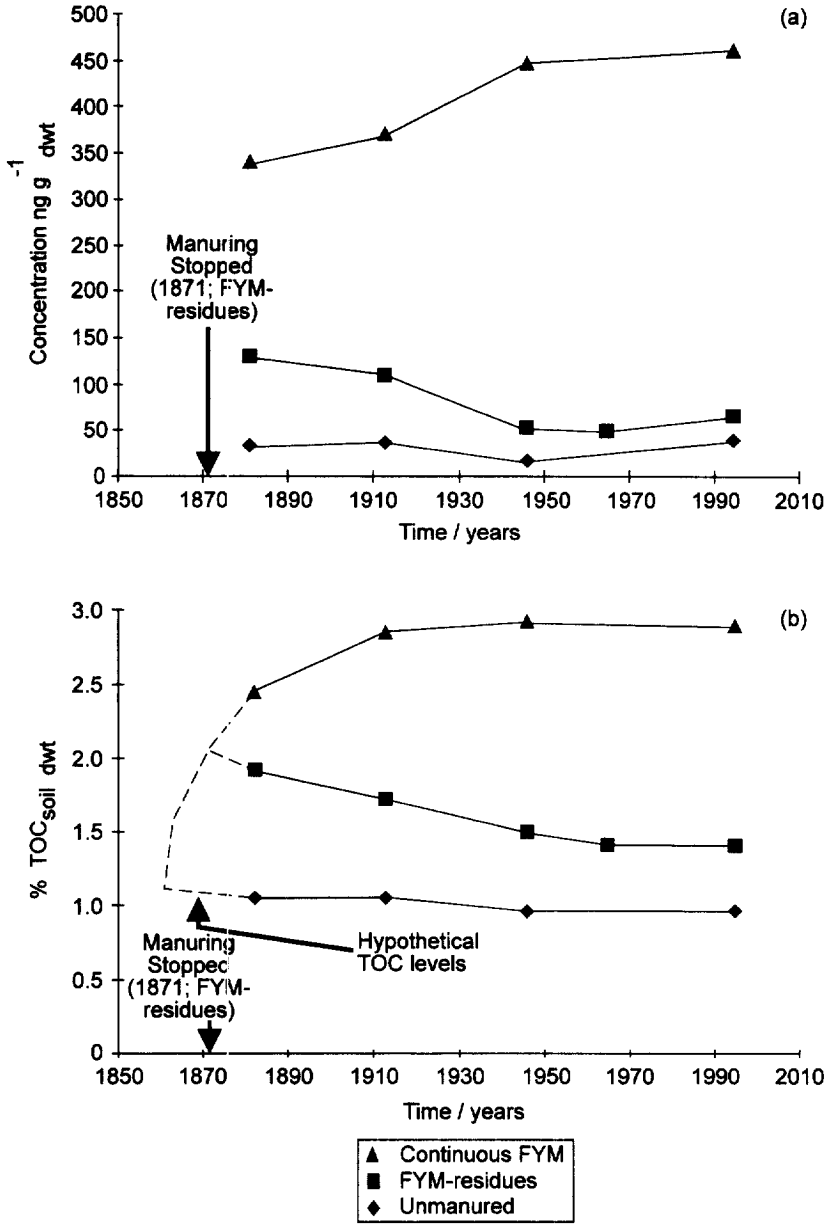

Fig. 6. Plots depicting the absolute concentration of: (a) 24-ethyl-5β-cholestan-3β-ol (**1c**) and (b) the total organic carbon content of the Unmanured, FYM-residues and Continuous FYM soils (the broken line represents a hypothetical predication of previous TOC levels).

all increase *vs* time, for each compound, in all three soils, the only exception is 24-ethyl-5α-cholestan-3β-ol (**3a**) in the Continuous FYM soil where a slight decrease in the 1995 sample was observed. The largest relative increases are seen in the FYM residues soil.

DISCUSSION

The results obtained will be discussed with reference to the lipids derived from the two extrapedological inputs, spring barley and FYM, and their expression within the soil. Furthermore, the behaviour of these lipids over time will be reviewed with particular emphasis on the longevity of specific biomarkers relating to the initial input of FYM. The discussion will end with an overview concerning the various archaeological implications raised by this study.

Evidence of extrapedological inputs

The aliphatic components do not exhibit sufficient specificity to allow unambiguous determi-

nation of variations in the quantity of either input. However, a number of interesting trends were observed. Although the wax ester profiles of the inputs are somewhat different, the major components in the barley (Fig. 1a) exist at a concentration an order of magnitude greater than of those in FYM (Fig. 1b), however, despite containing a greater abundance of wax esters on a dry weight basis, only the barley stubble and roots are ploughed back into the soil. We have estimated that *approximately* 28, 44 and 84 $t_{carbon}\ ha^{-1}$ from crop dry matter has been ploughed in on the Umanured, FYM-residues and Continuous FYM treatments respectively. In addition ca. 56 and 400 $t_{carbon}\ ha^{-1}$ from FYM dry matter has been added on the FYM-residues and Continuous FYM treatments respectively. The order of increase in total wax ester concentration (Unmanured < FYM-residues < Continuous FYM) could be attributed to the identical order of increasing crop yields for any particular year (Warren and Johnston, 1967). However, the low abundance of wax esters is not surprising given that only the crop stubble and roots are ploughed back into the soil. The distributional shift to longer homologues, with time, is most likely the result of *in situ* transesterification combined with selective degradation of the shorter homologues, and their constituent *n*-alkanol and fatty acid moieties (Jambu *et al.*, 1995). This would also explain why the C_{26} *n*-alkanol component of the soil wax esters is less abundant compared with those from the two extra-pedological inputs.

Both inputs exhibit similar *n*-alkanol distributions although, as with the wax esters, those in the barley are far more abundant on a dry weight basis (Fig. 2a and b). Hence, while initially it might be expected that these homologues will dominate the *n*-alkanol components of the soil, a number of longer chain *n*-alkanols occur in the soil samples that were not observed to any great extent in the two inputs. Likely sources of these compounds could be microbial reduction of long-chain fatty acids as suggested by Jambu *et al.* (1993) and/or homologues released *via* hydrolysis of previously transesterified wax esters, i.e. old carbon. The *n*-alkanols exhibit a lower abundance in the samples from 1913 and 1965 compared with those taken in other years. Both of these soils were sampled in October [1882 (Feb), 1946 (Aug) and 1995 (May); Poulton, pers. comm.], therefore, the differences in abundance of *n*-alkanols may result from differing degrees of assimilation or localised differences in soil organic matter content. Overall, there is probably no significant change in the *n*-alkanol content with time for the sequence of samples taken for the different treatments. It is interesting to note the dominance of C_{22} over C_{24} in all of the fractions except those from FYM-residues 1995 and Continuous FYM soil from 1946 and 1995 (Fig. 2n, k and o). The relative dominance of the C_{22} component is also seen in the FYM distribution (Fig. 2b), whilst, conversely the C_{24} homologue is more abundant in the barley distribution (Fig. 2a). This implies that most of this component is derived from the FYM input. Although the Unmanured treatment has received no FYM since the start of the experiment possible pre-experimental recalcitrant compounds might still affect experimental distributions. Certainly it would appear that the inclusion of barley dry matter only had a qualitative effect on three distributions, namely: FYM-residues 1995, Continuous FYM 1946 and Continuous FYM 1995. The total concentration of *n*-alkanols varies in the order Unmanured < FYM-residues < Continuous FYM, which is again analogous to the trend observed for the wax esters and can be attributed to increased crop yields (Warren and Johnston, 1967).

Similar distributions are noted for the even carbon number *n*-alkanoic acids of both barley and FYM (Fig. 3a and b). The former, however, possesses a greater abundance of *n*-hexadecanoic acid (C_{16}) and *n*-octadecanoic acid (C_{18}) whilst in the latter, higher homologues are more abundant, especially those $>C_{28}$ chain length. The *n*-alkanoic acids in the Unmanured and FYM-residues soils exhibit appreciable variation with time, whilst there is an apparent overall increase in the abundance of the longer homologues in the soils of the Continuous FYM soil. This can be explained by the addition of manure to the latter soil series with *n*-alkanoic acids from the manure off-setting any losses due to uptake and assimilation by soil microbiota. Samples from the 1995 soils clearly exhibit the result of selective degradation of short-chain *n*-alkanoic acids and similar trends have been observed in studies of a hydromorphic forest podzol (Amblès *et al.*, 1994). C_{32} $\beta\beta$ hopanoic acid (**12**) is a diagenetic degradation product of bacteriohopanetetrol, a structural membrane component in bacteria (van Dorsselaer *et al.*, 1974). Figure 5b shows the variation in concentration of this compound for the three treatments. In the Continuous FYM soil the concentration is maintained through time whilst in the FYM-residues soil there is a reduction in overall concentration, the difference in initial concentration between the FYM-residues and Continuous FYM soils is most likely related to sampling. In the Unmanured soils C_{32} $\beta\beta$ hopanoic acid (**12**) remains below the level of detection throughout the experiment. Since C_{32} $\beta\beta$ hopanoic acid (**12**) is present in FYM in significant amounts we can attribute the presence of this compound to successive manure applications. The decrease in the concentration of C_{32} $\beta\beta$ hopanoic acid (**12**) observed for the FYM-residues series is analogous to the trend seen for 24-ethyl-5β-cholestan-3β-ol (**1c**) which is also derived from the manure. The

lack of detectable C_{32} $\beta\beta$ hopanoic acid (**12**) in the Unmanured samples provides strong evidence for it deriving from the bacteria associated with manuring.

Analysis of the sterols and triterpenols (Fig. 4) reveals more significant differences between the Unmanured and Continuous FYM soil than are observed for the aliphatic components. The most profound difference is the increase in both number and concentration of stanols in the Continuous FYM soil with the FYM-residues soil containing a higher abundance of stanols than in the Unmanured soil but lower than those in the Continuous FYM soil. The Unmanured soil is dominated by the phytosterols, 24-methylcholest-5-en-3β-ol (**4b**), 24-ethylcholest-5,22-dien-3β-ol (**4e**) and 24-ethylcholest-5-en-3β-ol (**4c**) with somewhat smaller amounts of taraxerol (**9**), β-amyrin (**10**) and lupeol (**11**), and apart from taraxerol all are present in the more complex FYM-residues and Continuous FYM soils. None of the components detected can be considered specific biomarkers of barley. However, applications of FYM may be identified through the analysis of stanol components, in particular, 5β-stanols.

The *n*-alkanes detected in the two inputs and the modern-day soils from the three treatments are all similar, showing a strong odd over even predominance. Further evidence for a major input of FYM can be drawn from the fact that the modern-day soils all exhibit distributions with C_{31} maxima. This is a characteristic shared with the FYM distribution but not the barley distribution which has a C_{33} maximum. Paraffin wax, used in the sealing process of the archived soils, has most probably caused the significant changes to the *n*-alkane distributions in these soils. Using stable carbon and radiocarbon isotope measurements the original *n*-alkane distributions might be reconstructed (Lichtfouse and Eglinton, 1995).

Biomarker expression, survival and the archaeological implications

Whilst the addition of manure to the soil has undoubted effects on the lipid content (be they primary, or secondary due to enhanced plant and possibly microbial activity), they appear too common to be of use in the detection of manuring. 5β-Cholestan-3β-ol (**1a**) is a product of microbial reduction of cholest-5-en-3β-ol (**4a**) in the mammalian gut (Macdonald *et al.*, 1983; cf. 5α-cholestan-3β-ol (**3a**) as the natural product in the soil environment). This compound has previously been used in the detection of human faecal material in sewage pollution studies (Hatcher and McGillivray, 1979; Müller *et al.*, 1979; Brown and Wade, 1984; Readman *et al.*, 1986), and to detect inputs into soils and sediments at archaeological sites (Knights *et al.*, 1983; Pepe *et al.*, 1989; Pepe and Dizabo, 1990; Bethell *et al.*, 1994; Evershed and Bethell, 1996). As mentioned before, this approach has been successfully extended to include the use of the C_{29} 5β-stanol homologue to monitor manure application to modern day soils (Evershed and Bethell, 1996; Evershed *et al.*, 1997). Extending this technique to cover the higher homologue is important since ruminant mammals ingest high quantities of 24-ethylcholest-5-en-3β-ol (**4c**) through the consumption of vegetation. Hence, the reduction product 24-ethyl-5β-cholestan-3β-ol (**1c**) is a compound which may be utilised in the detection of manure from cattle and other herbivores. It can be seen from the results obtained thus far that the 5β-stanols are important steroidal components of soils subjected to manuring (Fig. 4). The study concerning the variance of 24-ethyl-5β-cholestan-3β-ol (**1c**) abundance over time clearly shows that whilst 5β-stanols will degrade within the environment of an active agricultural soil, the concentration in a previously manured soil remains greater than that of the background, represented by the Unmanured treatment, for at least 124 years (Fig. 6a). The actual trends observed are analogous to those published by Jenkinson and Johnston (1977) in a study of total organic carbon in soils from the same treatments and a more recent study of TOC performed in our laboratory (Fig. 6b). The "smoother" trend observed in these studies originates from the bulk nature of the total organic matter.

As mentioned in the introduction, understanding the changes in vegetation and/or land use are of significant importance for archaeological and quaternary environmental studies. Information obtained on possible application of manure to the soil has an important role to play in the spatial identification of site limits, field systems and their utilisation. The results obtained during this investigation show the 5β-stanols to be highly resistant biomarkers of manuring. Since a clear signal was detectable in the FYM-residues soil after 124 years of continual cropping and ploughing we would anticipate that such a signal would persist for a substantially longer period in less disturbed environments. This appears to be confirmed by our studies of fossil and undisturbed agricultural soils associated with Bronze Age settlements in the Orkney Islands (Simpson *et al.*, in press) and Pseira Island, situated off the Northeast coast of Crete, Greece (Bull *et al.*, 1995). Thus, 5β-stanol distributions provide a highly reliable criteria on which to base assessments of manure inputs. However, it must be stressed that such analyses may well be site specific since, due to different types of manure that might be utilised and variations in the background of 5β-stanols.

CONCLUSIONS

Fractionated total lipid extracts from soils of three Hoosfield Spring Barley plots, together with archived samples from the same plots (from 1882, 1913, 1946 and 1965), and the two major soil inputs, spring barley and farmyard manure were studied to examine the expression of the latter two inputs in the soil samples. Aliphatic components exhibited a number of trends giving insights into the process of diagenesis.

(i) Soil wax ester distributions were not dominated by either input. Evidence supports the action of *in situ* transesterification processes coupled with selective degradation exhibiting an inverse relationship to homologue chain length.

(ii) Soil *n*-alkanol distributions are influenced predominantly by the FYM input although some higher homologues are most likely derived from microbial and hydrolytic processes within the soil environment.

(iii) Soil *n*-alkanoic acids exhibited appreciable variation with time, the Continuous FYM treatment being the only soil to show an overall increase in homologue abundance, thus emphasising the important effect of FYM on the composition of these carboxylic acid distributions

Study of the survival of a number of isoprenoid biomarkers also provided new insights.

(iv) The bacterially derived biomarker C_{32} $\beta\beta$ hopanoic acid (**12**) was introduced to the soil as a constituent of manure and appears to provide an independent means of assessing the contribution of the manure related bacterial population.

(v) Assessment of faecal biomarkers, i.e. 5β-stanols, revealed that by measuring the absolute concentration of 24-ethyl-5β-cholestan-3β-ol (**1c**) a viable signal could be detected in a soil which had received no manure for 124 years. The residence of a signal indicative of manuring in an active, agricultural soil supports the use of this technique on older, less exposed sediments in a wide range of archaeological studies.

Associate Editor—**P. Farrimond**

Acknowledgements—Mr J. F. Carter and Mr A. R. Gledhill are thanked for their help with GC/MS analyses and the use of the NERC Mass Spectrometry Facilities (Grant: GR3/2951, GR3/3758. FG6/36101) is gratefully acknowledged. This project was undertaken whilst the authors were in receipt of a NERC Grant GR3/9578 to Dr R. P. Evershed and a Scholarship from the School of Chemistry, University of Bristol, to Mr I. D. Bull which are gratefully acknowledged. JACR receives grant aided support from the Biotechnology and Biological Council of the United Kingdom.

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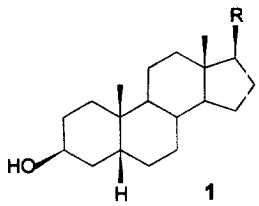
APPENDIX



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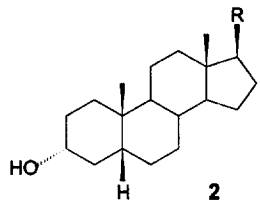

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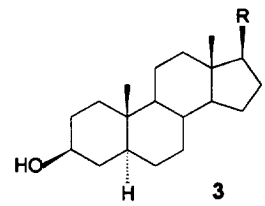

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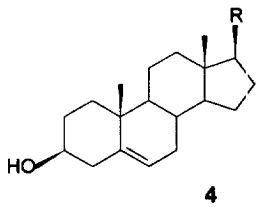

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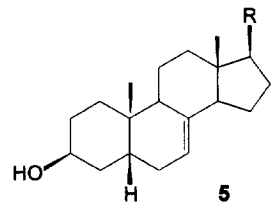

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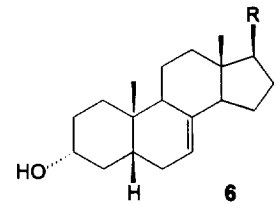

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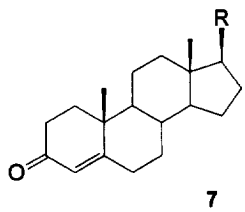

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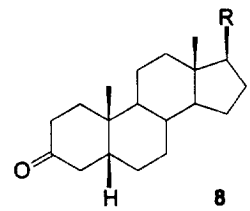

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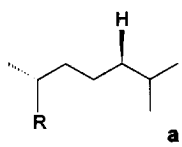

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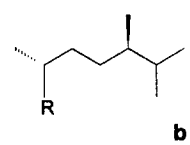

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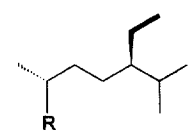

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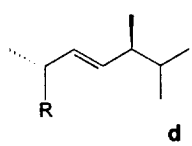

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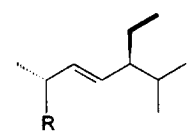

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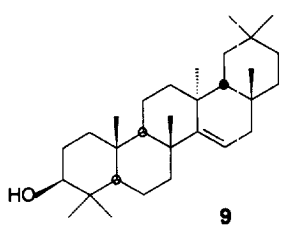

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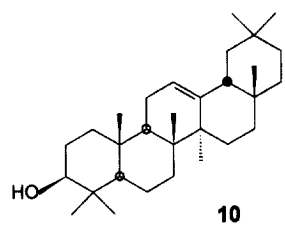

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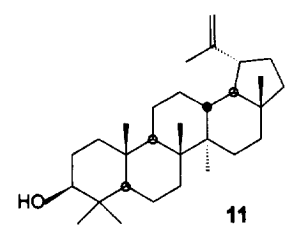

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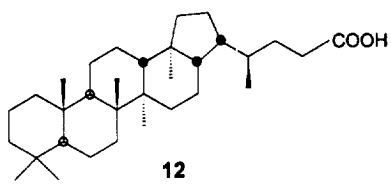

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